



## Short communication

## Pathobiology of triple reassortant H3N2 influenza viruses in breeder turkeys and its potential implication for vaccine studies in turkeys

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## ABSTRACT

Triple reassortant (TR) H3N2 influenza viruses have been isolated from turkeys in the United States since 2003. These TR H3N2 virus infections have been associated with drastic declines in egg production in breeder turkeys although co-infection with multiple agents could have been responsible for exacerbating the clinical signs. In this study, we experimentally confirmed that TR H3N2 influenza virus alone can cause drastic reduction/complete cessation of egg production and pathology of the reproductive tract in 26-week-old breeder turkeys. We confirmed high levels of virus replication and abundant distribution of avian specific  $\alpha 2,3$  sialic acid-galactose receptors in the oviduct of these turkeys. Although 2–6-week-old turkeys are routinely used for pathogenicity and vaccine protection studies, the low levels of viral shedding and asymptomatic infections in this age group often pose difficulty in interpretation of results. Our study shows that breeder turkeys should be used to assess the potential pathogenicity of TR H3N2 viruses and the viral titers and pathology of the oviduct as well as egg production data can be good measures of protection following in vivo challenge in vaccine efficacy studies.

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## 1. Introduction

Influenza viruses are negative sense segmented RNA viruses. The segmented nature of the genome facilitates genetic reassortments during co-infection of host with different influenza A viruses [1]. Wild aquatic birds are considered to be natural reservoirs of influenza viruses [2,3]. However, influenza viruses can infect a wide range of mammals and birds resulting in infections of varying severity. Wild bird isolates replicate poorly in some of their new or aberrant hosts. In some cases, these viruses can adapt well and replicate efficiently for a prolonged period of time in their new hosts and establish a stable lineage [4]. One of the major selective pressures for influenza viral infections is the availability of receptors on the cell surfaces of the hosts [5,6]. Mammalian influenza viruses preferentially bind to  $\alpha 2,6$  sialic acid (SA)-galactose (gal) terminated sialyloligosaccharides, whereas avian viruses prefer  $\alpha 2,3$ SA-gal terminated residues [4]. Pigs, however, express both  $\alpha 2,3$ SA-gal and  $\alpha 2,6$ SA-

gal receptors on their tracheal epithelium and are implicated as potential mixing vessels of avian and mammalian influenza viruses [7,8].

Triple reassortant (TR) H3N2 influenza viruses containing gene segments derived from recent human (HA, NA and PB1), swine (NS, NP and M) and avian (PB2, PA) influenza viruses [9,10] have become endemic in U.S. swine population, since their first isolation in 1998 [9]. Since 2003, the swine origin TR H3N2 viruses have been isolated from U.S. turkey populations [11,12]. In most cases, TR H3N2 viruses have been isolated from breeder turkeys with symptoms of moderate to drastic declines in egg production [11–13]. Experimental infection studies with these viruses demonstrated that they can infect and replicate in pigs, turkeys and quail [11]. Intraspecies transmission of these viruses was also reported in turkeys. However, these replication and pathogenicity studies with TR H3N2 viruses were done in 2–4-week-old birds [14] with no published reports of pathogenicity studies in breeder turkeys using TR H3N2 viruses.

In this study, we conducted pathogenicity studies in 26-week-old breeder turkeys to assess the effect of TR H3N2 virus infection on egg production. We determined the replication of the virus in different parts of the oviduct in addition to upper respiratory and digestive tracts. In addition, we determined the influenza receptor profile in the oviduct.

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## 2. Materials and methods

### 2.1. Viruses

Two TR H3N2 viruses used in this study, A/turkey/Illinois/04 (TK/IL/04) and A/turkey/Ohio/313053/04 (TK/OH/04), were isolated at Food Animal Health Research Program, Wooster, Ohio [12,13]. These viruses were passaged twice in Madin-Darby Canine Kidney cells (MDCK) for initial isolation and passaged one more time to make working stocks.

### 2.2. Pathogenicity of TK/IL/04 and TK/OH/04 viruses in breeder turkeys

Pathogenicity studies were undertaken in 26-week-old breeder turkeys by infecting two groups of 18 birds each with  $10^{6.5}$  TCID<sub>50</sub>/0.5 ml of virus through choanal route. One additional group of 12 turkeys were inoculated with sterile phosphate buffered saline (PBS) and served as negative controls. Tracheal and cloacal swabs were collected from the infected and control birds at 2 and 4 days post-infection (DPI). Individual swabs were placed in 2.0 ml of PBS containing gentamycin (1 mg/100 ml). The swab samples were vortexed for 10 s and centrifuged for 5 min at 1200 rpm. Two hundred microliters of the supernatant from the swab samples was used for RNA extraction using RNeasy Mini Kit (Qiagen, Valencia, CA) in accordance with the manufacturer's instructions. The RNA was eluted in 50  $\mu$ l of nuclease free water and 8  $\mu$ l of eluted RNA was used for quantitation by real-time RT-PCR (RRT-PCR) as previously reported [15]. For quantitation, swab samples were run together with known amounts of control viral RNA. We used RNA extracted from  $10^1$  to  $10^5$  EID<sub>50</sub>/0.2 ml dilutions of the TK/IL/04 and TK/OH/04 stock viruses, respectively, as standard controls. Standard curves were generated with those control viral RNAs and the amount of RNA in the swab samples was converted into EID<sub>50</sub>/ml by interpolation. High correlation ( $r^2 > 0.99$ ) between cycle number and dilution factors were observed with standard controls.

At 7 DPI, two infected turkeys from each group were euthanized and tissues (trachea, lungs, kidney, spleen, portions of small and large intestine, cecal tonsils and different parts of the oviduct) were collected and preserved in 10% buffered formalin for histopathology. Virus replication in reproductive tract of turkeys was assessed by collecting each of infundibulum, magnum, isthmus and uterus at 7 DPI in sterile PBS (5 g/1 ml ratio). Five grams each of infundibulum and isthmus and 25 g each of magnum and uterus were collected in 1 ml and 5 ml of sterile PBS, respectively. RNA extraction and viral titration by RRT-PCR were done as for tracheal and cloacal swabs using 200  $\mu$ l of tissue supernatant. The RNA was eluted in 50  $\mu$ l of nuclease free water and 8  $\mu$ l of eluted viral RNA was used for quantitation using RRT-PCR. As for the swab samples, the amount of RNA in the tissue samples was converted into EID<sub>50</sub>/0.2 ml (which is equal to EID<sub>50</sub>/g) by interpolation. Egg production data was monitored twice daily in the treatment and control birds for 1 week prior to and 3 weeks post-infection. All the birds were bled at 21 DPI and HI titers were determined [16]. The endpoint HI titer was defined as the last dilution of serum that completely inhibited hemagglutination.

### 2.3. Lectin immunostaining for receptors

We used different parts of the oviduct (magnum, isthmus, uterus) for studying the receptor profile by employing two specific lectins, Maackia amurensis agglutinin (MAA) for  $\alpha$ 2,3SA-gal receptors and Sambucus nigra agglutinin (SNA) for  $\alpha$ 2,6SA-gal receptors (DIG Glycan Differentiation Kit, Roche Applied Science, Mannheim, Germany). Due to the practical difficulties in getting

tissue sections, we did not include infundibulum for receptor studies. Paraffin embedded sections of the oviduct were deparaffinized and immersed in 3% hydrogen peroxide to eliminate the endogenous peroxidase activity. The sections were treated with blocking agent (DIG Glycan Kit, Roche) to avoid nonspecific staining and then incubated with digoxigenin (DIG)-labelled MAA or SNA (1  $\mu$ g/ $\mu$ l) at 4 °C overnight. After two washes in PBS, the sections were incubated with peroxidase-labelled anti-DIG FAb fragments (Roche Applied Science) for 1.5 h at 37 °C. Lectin binding was visualized using DAB (3,3'-diaminobenzidine-tetrahydrochloride) substrate (Roche diagnostics GmbH, Mannheim, Germany) and slides were counterstained with hematoxylin. Negative controls were incubated with PBS instead of lectin.

### 2.4. Histopathology and immunohistochemistry (IHC) for viral antigen

Tissues were fixed by submersion in 10% neutral buffered formalin, routinely processed, and embedded in paraffin. Sections were made at 5  $\mu$ m and were stained with hematoxylin and eosin (HE). A duplicate 4- $\mu$ m section was immunohistochemically stained by first microwaving the sections in Antigen Retrieval Citra Solution (Biogenex, San Ramon, CA) for antigen exposure. A monoclonal antibody (P13C11) specific for a type A influenza virus nucleoprotein, developed at Southeast Poultry Research Laboratory, was used as the primary antibody for a streptavidin-biotin-alkaline phosphatase complex-based IHC method as previously described [17].

## 3. Results

### 3.1. Replication and pathogenicity of TK/OH/04 and TK/IL/04 viruses in breeder turkeys

Pathogenicity studies were undertaken by infecting two groups of breeder turkeys with  $10^{6.5}$  TCID<sub>50</sub>/0.5 ml of either virus through choanal route. In turkeys inoculated with TK/IL/04 virus, only 3 out of the total 18 birds tested positive for viral RNA from tracheal and cloacal swabs at 2 DPI with the mean interpolated viral titers of 1.06 and 0.92 log<sub>10</sub> EID<sub>50</sub>/ml, respectively (Table 1). In the group inoculated with TK/OH/04 virus, we observed comparatively higher titers of 1.74 and 2.16 log<sub>10</sub> EID<sub>50</sub>/ml in the tracheal (4/18 positive birds) and cloacal swabs (7/18 positive birds), respectively, at 2 DPI. Similar trends were observed with swab samples collected at 4 DPI with slightly higher viral titers observed in TK/OH/04 infected birds. Viral titers in different parts of the oviduct were also determined. In the TK/IL/04 group, only infundibulum and magnum showed positive results with average titers of 1.07 and 0.19 log<sub>10</sub> EID<sub>50</sub>/g, respectively. The TK/OH/04 group showed comparatively higher viral titers in all four parts of the oviduct. The average viral titers in infundibulum was 3.76, magnum showed the highest titer of 5.84, and isthmus and uterus had titers of 4.96 and 5.23 log<sub>10</sub> EID<sub>50</sub>/g of the tissue, respectively. All the infected birds in both groups seroconverted as shown by the HI titers observed at 21 DPI (Table 1). The HI titers were higher in the TK/OH/04 group in comparison to the TK/IL/04 group (average titers of 11.9 log<sub>2</sub> and 4.8 log<sub>2</sub>, respectively).

The TK/IL/04 group maintained a steady egg production similar to the control group. However, the TK/OH/04 group showed a drastic decline in egg production at around 7 DPI, finally leading to a complete cessation of production at about 15 DPI (Fig. 1). Apart from the lowered egg production, we did not observe any other clinical signs in the infected birds from either treatment groups.

We collected tissues for microscopic examination from infected birds at 7 DPI. In both groups of birds, we observed only mild lesions in the trachea and lungs consisting of mild hyperplasia of the tra-

**Table 1**

Replication of A/turkey/Illinois/04 and A/turkey/Ohio/04 in 26-week-old breeder turkeys.

Virus	Samples	Virus detection <sup>a</sup>			HI titer <sup>b</sup>
		2 DPI <sup>c</sup>	4 DPI	7 DPI	
TK/IL/04	Tracheal swabs	1.06 ± 0.33 (3/18) <sup>d</sup>	0.90 ± 0.19 (3/18)	ns	4.8 ± 2.76
	Cloacal swabs	0.92 ± 0.18 (3/18)	0.91 ± 0.2 (4/18)	ns	
	Oviduct				
	Infundibulum	ns	ns	1.76 ± 1.26 (2/2)	
	Magnum	ns	ns	0.88 (1/2)	
	Isthmus	ns	ns	0	
	Uterus	ns	ns	0	
TK/OH/04	Tracheal swabs	1.74 ± 0.34 (4/18)	2.64 ± 0.58 (16/18)	ns	11.9 ± 0.24
	Cloacal swabs	2.16 ± 0.65 (7/18)	4.14 ± 1.01 (6/18)	ns	
	Oviduct				
	Infundibulum	ns	ns	3.76 ± 2.62 (2/2)	
	Magnum	ns	ns	5.84 ± 1.62 (2/2)	
	Isthmus	ns	ns	4.96 ± 1.96 (2/2)	
	Uterus	ns	ns	5.23 ± 1.23 (2/2)	

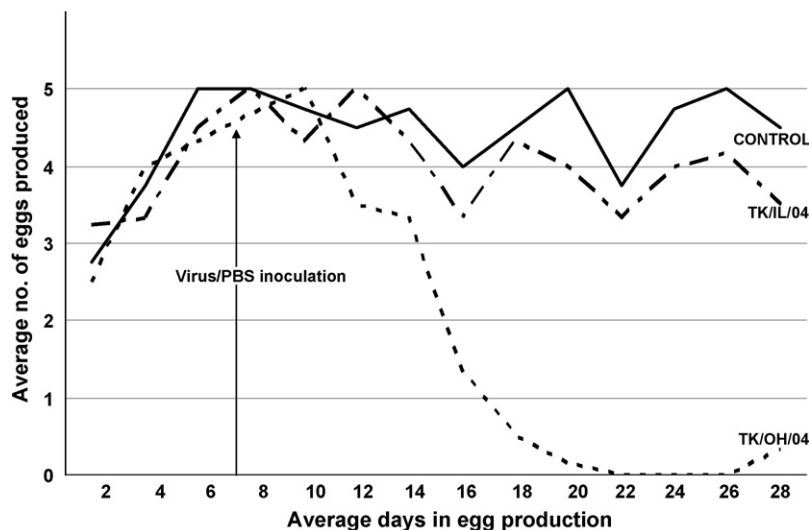
ns: Not sampled.

<sup>a</sup> log<sub>10</sub> 50% egg infectious dose (EID<sub>50</sub>)/ml of swab supernatant or EID<sub>50</sub>/g of oviduct tissues ± standard deviation determined by RRT-PCR.<sup>b</sup> log<sub>2</sub> hemagglutination inhibition (HI) titer of the antisera ± standard deviation.<sup>c</sup> Days post-infection.<sup>d</sup> Number of positives/total numbers tested.

cheal epithelium with mild lymphoplasmacytic infiltration, mild bronchitis and mild congestion of the lungs. Viral antigen was not detectable in the trachea and lungs of turkeys from either group. Mild lymphoid hyperplasia in the cecal tonsils and lymphoid atrophy in the spleen was observed, however, viral antigen was not demonstrable. In the intestines, mild infiltration of lymphocytes in the lamina propria was present mostly in the jejunum with no or infrequent viral antigen staining. In kidneys, mild to moderate multifocal tubule necrosis and associated lymphocytic interstitial nephritis was observed in birds from both groups, with viral antigen staining present in the tubule cells. The oviduct presented marked lesions in the TK/OH/04 infected birds. In these birds, degenerative and necrotic changes were found throughout the oviduct, with loss of cilia and degeneration and necrosis of the oviduct surface epithelium, and atrophy of the glandular epithelium. Viral antigen staining was demonstrated in the lining cells of all sections of the oviduct and was less evident in the glandular epithelium

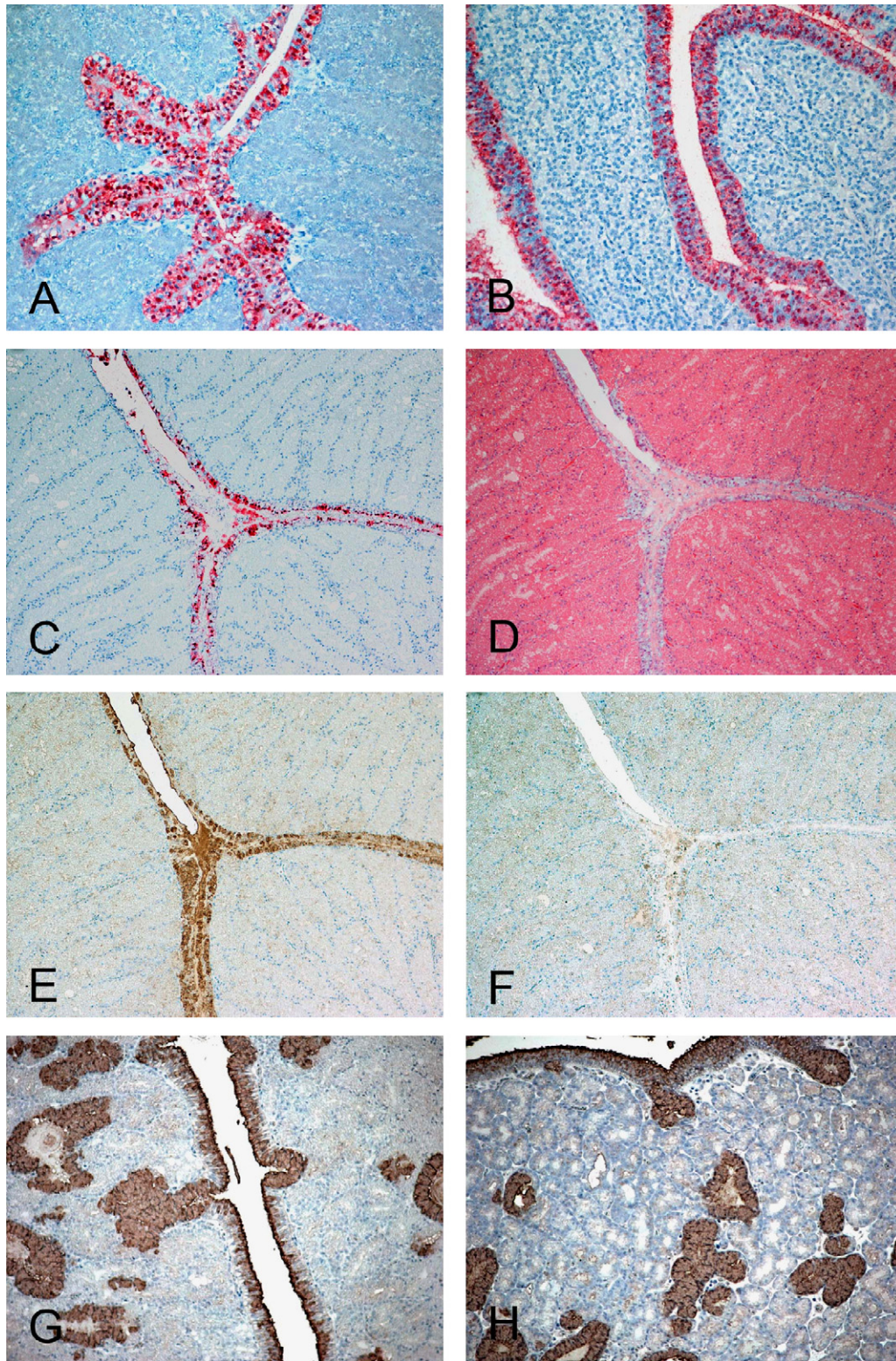
(Fig. 2 A–C). Surface epithelial cells of the magnum showed extensive necrosis and desquamation (Fig. 2D). Degenerative and diffuse necrotic changes were observed in the glandular epithelial cells of isthmus and uterus with accumulation of cellular debris between folds and within the lumen. Multifocal lymphocytic infiltration of the glands was also observed. In the TK/IL/04 infected group, mild to moderate degeneration of epithelial cells, mild atrophy of oviduct glands, and edema of the submucosa were observed with mild staining for viral antigen.

We also studied the type and distribution of receptors in the oviduct of the breeder turkeys using immunostaining employing specific lectins. We observed an abundance of α2,3SA-gal terminated sialyloligosaccharide receptors in different parts of the oviduct (Fig. 2E, G and H). Staining for these receptors was very marked on the surface epithelium of all sections of the oviduct and less common in the glandular epithelium. We observed little or no specific staining for the α2,6SA-gal receptors (Fig. 2F). In duplicate



**Fig. 1.** Egg production data from infected and uninfected control breeder turkeys for a period of 28 days (from 1 week prior to infection to 3 weeks post-infection). The X-axis represents the average days in egg production, 2 days being represented as one unit. The Y-axis represents the average egg production during the 28-day period, average no. of eggs produced by each bird in 2 days being represented as one unit. The arrow represents the day of virus inoculation. The solid line represents egg production data from the control birds that were inoculated with phosphate buffered saline (PBS) and two different dashed lines represent the data from A/turkey/Ohio/313053/04 (TK/OH/04) and A/turkey/Illinois/04 (TK/IL/04) infected birds, respectively.





**Fig. 2.** Oviduct sections from 26-week-old breeder turkeys. Sections A–C stained by IHC methods to detect viral antigen from TK/OH/04 infected birds. The red color indicates viral antigen staining. Sections E, G and H stained with Maackia amurensis agglutinin (MAA) to demonstrate  $\alpha 2,3$ SA-gal receptors. The brown color indicates the presence of  $\alpha 2,3$ SA-gal receptors. Positive staining for both virus (from TK/OH/04 infected birds) and receptors is observed on the lining epithelium of the magnum (A and G respectively), uterus (B) and isthmus (H) (magnification 200 $\times$ ). Magnum showing correlation between Maackia amurensis agglutinin (MAA) staining for  $\alpha 2,3$ SA-gal receptors (E) and IHC staining for viral antigen (C) from TK/OH/04 infected birds. Hematoxylin and eosin stained section of magnum from TK/OH/04 infected birds demonstrating histological lesions (D). Magnum section stained with Sambucus nigra agglutinin (SNA) to demonstrate  $\alpha 2,6$ SA-gal receptors (F).



sections of isthmus that were stained for receptors as well as viral antigen from TK/OH/04 infected birds, we observed that the lining epithelial cells of isthmus that stained positive for MAA gave positive results for viral antigen staining (Fig. 2C and E). Due to extensive loss of epithelia in other sections of the oviduct, we could not duplicate this result for other oviduct sections.

#### 4. Discussion

H3N2 avian influenza infections in turkeys may be asymptomatic or cause clinical disease with symptoms ranging from varying degrees of depression, anorexia, mild to severe respiratory illness, diarrhea, sinusitis, edema of head and face, cyanosis, decline in egg production, egg shell abnormalities, decrease in egg hatchability and occasional mortality [12,18,19]. These symptoms can manifest themselves alone or in any combination. In breeder turkeys, low pathogenic influenza A infections almost always result in decreased egg production. Several previous reports document drops in egg production in turkeys due to H1N1 [20,21], reassortant H1N2 [18], and H3N2 viruses [11,12,22] from different parts of the world. TR H3N2 viruses have been associated with sudden declines in egg production in breeder turkeys within the U.S. [11,12]. However, although influenza viruses were isolated or serological evidence of influenza infections were reported in those flocks, it is possible that co-infection with multiple agents could be responsible for the observed reduced or complete cessation of egg production. To date, there are no reports of pathogenicity studies in breeder turkeys using TR H3N2 viruses. In this study, we used two turkey TR H3N2 viruses, TK/OH/04 and TK/IL/04, for their replication and pathogenicity in breeder turkeys. These two viruses were of particular interest as they had been isolated from turkey flocks showing symptoms of lowered egg production. We observed that TK/OH/04 was better adapted to turkeys based on the higher virus replication observed by RRT-PCR in tracheal and cloacal swabs and oviduct samples. Interspecies transmission studies with TK/OH/04 and TK/IL/04 from turkeys to pigs and vice versa showed that only TK/OH/04 virus was capable of transmission in both directions [14]. Thus, though these two viruses are antigenically almost identical and show  $\geq 99\%$  sequence similarity in all 8-gene segments [13], individual viruses vary in their replication and transmission characteristics.

We observed that TK/IL/04 and TK/OH/04 viruses replicated to higher titers in the oviduct of the breeder turkeys as compared to the respiratory or digestive tract. The TK/OH/04 infected turkeys showed high viral titers in all four parts of the oviduct with the lowest titers in the infundibulum. The extensive replication of the TK/OH/04 virus in different parts of the oviduct altering its physiology and anatomical architecture appears to be responsible for the drastic decline in egg production. Microscopic examination of the TK/OH/04 virus infected breeder turkey tissues revealed degenerative and necrotic changes of the surface epithelia of the oviduct as well as necrosis and severe atrophy of the glandular epithelium. In contrast, in the TK/IL/04 group, the virus replication was noticeable only in the infundibulum and magnum. Histopathology also revealed milder lesions in the oviduct of these birds. This study alone does not provide the answers for higher viral titers and severe lesions observed with TK/OH/04 virus infection in the oviduct, however it confirms that TR H3N2 influenza virus alone, without any concurrent infections, can lead to drastic decline in egg production in breeder turkeys.

We demonstrated the predominance of  $\alpha 2,3$ SA-gal receptors in the magnum, isthmus and uterus of the oviduct of the breeder turkeys (Fig. 2E, G and H). In these oviduct sections, the staining for  $\alpha 2,6$ SA-gal receptors were very few or none (Fig. 2F). The direct correlation between the presence of  $\alpha 2,3$ SA-gal receptors

and viral antigen in duplicate sections of the oviduct indicates that the viruses might have utilized these receptors for virus–cell interactions (Fig. 2C and E). Further binding assay studies using epithelial lining of the oviduct from breeder turkeys are necessary to validate these findings. Previous results on intraspecies transmission of TK/OH/04 virus within chickens, ducks and turkeys by our group had led us to speculate that these viruses might predominantly use  $\alpha 2,6$ SA-gal receptors for cell attachment and entry [14]. This hypothesis is weakened in light of our recent findings (unpublished data). Studies using two recent 2005 turkey TR H3N2 viruses revealed that these viruses can replicate within turkeys, chickens and ducks based on seroconversion observed in infected and contact control birds, although viral titers determined by RRT-PCR from tracheal and cloacal swabs of chickens and ducks were low or none. Intraspecies transmission studies showed that these two isolates transmitted within turkeys and ducks, while only one of them transmitted among chickens. Based on the exact match between the distribution of viral antigen and  $\alpha 2,3$ SA-gal receptors on the oviduct of breeder turkeys observed in this study as well as the recent intraspecies transmission data, we believe that these viruses utilize  $\alpha 2,3$  SA-gal receptors for infection. However, it should be re-emphasized that influenza infections depend on multiple factors of the host and the viruses other than receptors.

Routinely, 2–6-week-old turkeys are used in pathogenicity and vaccine efficacy studies against influenza [23,24] due to ease of handling and management of the birds. Reduction in virus shedding from trachea or cloaca following challenge infection is used as the main indicator of protection in vaccine studies in the absence of morbidity or mortality. Previous results [14] as well as our recent characterization studies (unpublished data) show that TR H3N2 viruses replicate to very low titers in the respiratory or digestive tract of these birds. Furthermore, virus inoculation in these birds almost always results in asymptomatic infections which make it difficult to determine the efficacy of the vaccine. On the other hand, use of breeder turkeys in vaccine trials might have several advantages. Pre- and post-challenge egg production data could be a reliable way of assessing vaccine protection. Also, viral titers from the oviduct of these birds have been consistently higher than those from tracheal or cloacal swabs. Thus, a comparison between vaccinated and non-vaccinated groups following challenge infection based on the viral titers from oviduct becomes easily comprehensible. In addition, as only breeder turkeys are routinely vaccinated against influenza among turkeys in the U.S., their use as experimental animals in vaccine studies will help in the development of better vaccines for that target group.

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